Supplement to:

Chronic Dietary Exposure to a Low-Dose Mixture of Genistein and Vinclozolin Modifies the Reproductive Axis, Testis Transcriptome and Fertility

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Supplemental Material, Table 1. Effects of treatments on sperm kinematic variables in rats aged 80 days

Characteristics*		Treatment (mg/kg bw/day)					
	Control	g(1)	G(10)	v(1)	V(30)	g(1)+v(1)	G(10)+V(30)
VSL (µm/s)	83±4	69±2††††	71±2††††	70±3††††	64±3††††	63±4††††	67±3††††
VAP (µm/s)	132±4	120±2††††	114±2††††	123±2†	115±3††††	116±3††††	122±2††
VCL (µm/s)	308±12	254±5††††	254±5††††	263±6††††	247±5††††	257±10††††	251±6††††
ALH (µm)	16.4±0.5	15.1±0.9	16.3±0.5	17.1±0.7	15.0±0.8††	15.1±2.0	14.0±1.3
BCF (Hz)	26.7±0.9	23.7±0.4†††	24.5±0.5††	24.1±0.4†††	25.8±0.7	24.9±0.9	25.3±0.8
STR (%)	63.8±1.8	58.0±1.4†††	63.4±0.9	57.7±1.7††	57.0±1.8†††	56.0±2.2†††	56.4±2.0†††
LIN (%)	27.4±0.7	27.7±0.7	29.1±0.7	27.6±0.8	26.9±0.8	26.0±0.9	27.8±0.9

††p<0.05, †††p<0.01, ††††p<0.001

VSL: straight line velocity, VAP: average path velocity, VCL: curvilinear velocity, ALH: lateral head displacement, BCF: beat cross frequency, STR: straightness of trajectory and LIN: linearity of trajectory.

Supplemental Material, Table 2. Primers for qRT-PCR

ADAM7	LEFT PRIMER	164	20	60.07	50.00	3.00	2.00	11.00	agcaaggacccaagaggaat
	RIGHT PRIMER	272	20	60.03	50.00	4.00	2.00	10.00	tctgcatgggggatctttag
ERABP	LEFT PRIMER	42	20	59.87	55.00	5.00	2.00	12.00	gaccactgtgtgctggagaa
	RIGHT PRIMER	157	20	59.73	60.00	2.00	1.00	12.00	gtcaggtagtcggtggcttc
NFASC	LEFT PRIMER	90	20	59.97	50.00	5.00	0.00	10.00	aagccacaacagttcccatc
	RIGHT PRIMER	206	20	60.11	60.00	4.00	2.00	9.00	gaggactctccgtggtggta
IL21R	LEFT PRIMER	368	20	60.20	50.00	3.00	0.00	10.00	gaagaaccatctgccttgga
	RIGHT PRIMER	476	20	59.85	45.00	5.00	0.00	9.00	tttcttgaagttcccgctgt
ALB	LEFT PRIMER	5	20	59.97	50.00	5.00	0.00	11.00	aattggcaacagacctcacc
	RIGHT PRIMER	124	20	60.07	55.00	4.00	0.00	10.00	tggagatagtggcctggttc
TESTIN	LEFT PRIMER	60	20	60.29	55.00	4.00	0.00	11.00	catctgaggagcagcagaca
	RIGHT PRIMER	168	20	59.96	55.00	4.00	0.00	10.00	actgggatctggtgttggag
MUSK	LEFT PRIMER	23	20	60.22	55.00	4.00	0.00	10.00	tgccacccgagtctatcttc
	RIGHT PRIMER	122	20	60.36	55.00	6.00	0.00	10.00	ggctgcagtccataggagaa
Kremen1 A	LEFT PRIMER	206	20	60.35	45.00	6.00	2.00	10.00	ttctctgatcgcatcaacca
	RIGHT PRIMER	292	20	59.97	60.00	6.00	1.00	11.00	gatagcaggcctctcctgtg
Kremen1 B	LEFT PRIMER	31	20	60.22	55.00	3.00	1.00	10.00	ccccctggtccttacagttt
	RIGHT PRIMER	139	20	60.07	55.00	3.00	2.00	11.00	gggtgctatcccttgtgaga
CRISP1	LEFT PRIMER	257	20	59.97	45.00	6.00	3.00	9.00	ccaattgagacgaacggttt
	RIGHT PRIMER	354	20	60.50	45.00	3.00	3.00	11.00	acctgtttgcccatttctga
LPL	LEFT PRIMER	19	20	59.84	50.00	4.00	1.00	10.00	ttctgtgccagggagaaagt
	RIGHT PRIMER	129	20	59.73	50.00	3.00	2.00	10.00	ttggtttgtccagtgtcagc

Supplemental Material, Table 3. Correlation matrix of the different gene modification ratios between the treatments and the controls

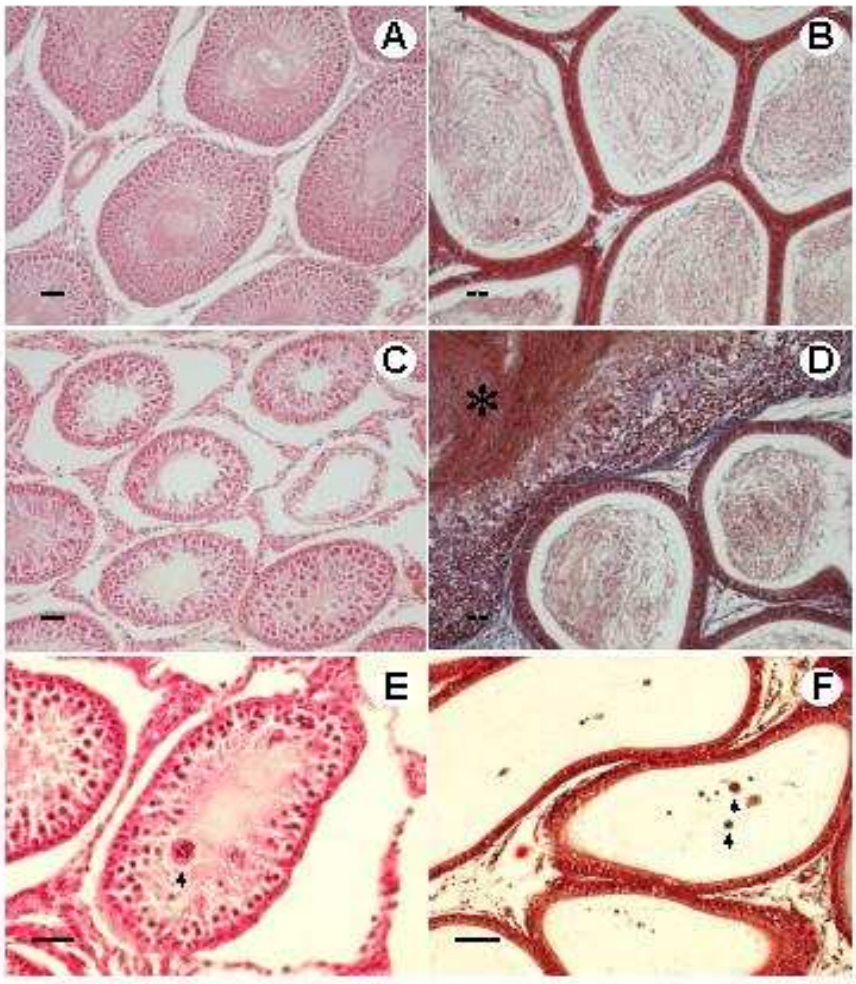
	g(1)/C	G(10)/C	v(1)/C	V(30)/C	g(1)+v(1)/C
G(10)/C	0.212				
v(1)/C	-0.070	0.087			
V(30)/C	0.060	0.086	0.771		
g(1)+v(1)/C	0.268	0.156	0.707	0.818	
G(10)+V(30)/C	0.431	0.306	0.463	0.479	0.656

Supplemental Material, Table 4. Effects of dietary genistein (G) doses and intermediate or low-doses vinclozolin (V) administered by gavage on the rodent male reproductive system and fertility: comparison of data in the literature with that from our study according to exposure period and dose. * g: gestational, pn: perinatal or lactational, p: pubertal, pp: peripubertal, ga: gestation to adulthood, a: adulthood; ** F0 ♀ exposed for two weeks before mating with unexposed ♂; *** F0 ♂+♀exposed for 10 weeks before mating between exposed ♂ and ♀; † LE: Long-Evans, SD: Sprague Dawley, W: Wistar; §: delay to preputial separation

Compound(s)	G	G	G	G	G	G	G	V	V	V	V	V	V	GV
Reference	Wisniewski 2003	Fielden 2000	Nagao 2001	Lee 2004	Delclos 2001	Roberts 2000	Our study	Gray 1999	Monosson 1999	O'Connor 2002	Shin 2006	Matsuura 2005	Our study	Our Study
Animal model:														
R:rat/strain† M:mice	R/LE	M	R/SD	M	R/SD	R/SD	R/W	R/LE	R/LE	R/SD	R/SD	R/SD	R/W	R/W
	*F0**F1:g/pn*	*g/pn*	*pn*	*pp*	*g to pp*	*ga*	*ga*	*g/pn*	*pp*	*a*	*p to a*	*F0***F1:ga*	*ga*	*ga*
*Exposure period **	GD0-PND21	GD12-PND20	PND1-PND5	PND21-PND56	GD7-PND50	GD17-PND70	GD1-PND80	GD14-PND3	PND22-PND56	PND70-PND85	PND42-PND70	GD1-PND70	GD1-PND80	GD1-PND80
Dose (mg kg^{-1} day^{-1})	~0.8(in diet)	0.5 10	12.5	2.5 5	0.5 10	0.3	1 10	3 25	10 30	10	~3 50	~2-6(in diet)	1 30	1/1 10/30
Necropsy period	PND70	PND105	PND84 PND98	PND56	PND50	PND70	PND85	PND107 PND350	PND56	PND85	PND71	~PND71	PND85	PND85
Endpoints														
Anogenital distance	=	= ↓			=		= =	↓ ↓					↓ ↓	↓ =
Nipple retenion/development								↑ ↑				↑ (6% vs 0%)		
Immature penis/PPS§	Yes		No		No No		No Yes		No No				Yes No	Yes No
Hypospadias							No No	No No					No Yes	No ±Yes
Undescended testis							No ±Yes	No No					No ± Yes	No No
Epididymal granuloma							No No						No No	Yes No
Body weight (Bw)		= =	↓	= =	= =	=	↑ ↑	= =	= =	=	= =		= =	= ↑
Bw gain			=	= =	= =	=	= =	= =	= =	=	= =		= =	= =
Testis w	=	= ↑	=	= =	= =	=	= =	= =	= =	=	= =	=	= =	= ↑
Epididymis w	=		=	= =	= =	=	↓ ↓	= =	= ↓	=	= ↓	=	↓ ↓	↓ ↓
Seminal vesicles w	=	= =	=		= =		= =	= =	= =	=	= =	=	= ↓	↓ ↓
Ventral prostate w	↑		↑	= =	= ↑		= =	= ↓	= =	=	= =	=	= ↓	= ↓
Sperm production	=	= =	=	= =	= =	=	= ↓	= =	= =		= =	=	= ↓	↓ ↓
Sperm prog. Motility		= =		= =			↓ =				= =	=	↓ ↓	↓ ↓
Sperm motion param.		= =		= =			↓ ↓						↓ ↓	↓ ↓
FSH LH	↓					= =	= = = =		↑(LH)↑(LH)	= =	= = = =	= =	= = ↑↑	↑ = ↑ =
						=	= = ↓ =			= =	= = = =	=	= = = =	= ↓ ↓ =
Testosterone. Estradiol								= (T) =(T)	=(T) =(T)					
Mating			=				= =					=	↓ =	↓ =
Fertility		= (*FIV*) ↑	=				= =	= =				=	= ↓	= =
Litter size			=				↓ ↓					=	↓ ↓	↓ ↓
Postimplantation loss			=				↑ ↑					=	↑ ↑	↑ ↑

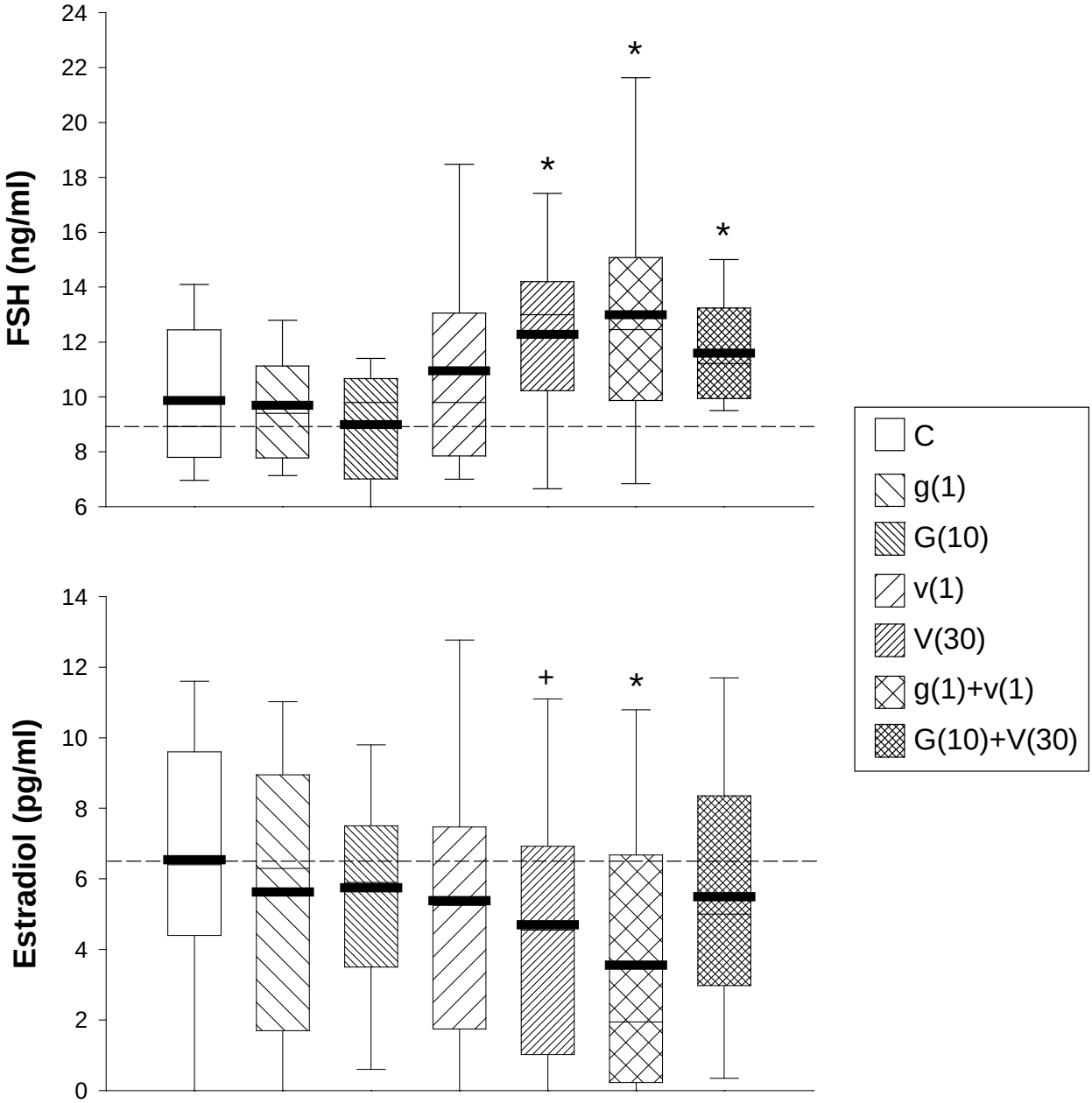
Supplemental Material, Figure 1. Adult (PND80) testis and epididymis histology in the control group (A and B, respectively) and in the group exposed to the low-dose genistein+ vinclozolin mixture, g(1)+v(1) (C-E and D-F, respectively).

Normal spermatogenesis (A) and epididymal sections with a normal epithelium and a lumen full of spermatozoa (B) were observed for most control rats. In the g(1)+v(1) group, sections of seminiferous tubules revealed various degrees of abnormal spermatogenesis including tubule degeneration with detachment of germ cells from epithelium (C); multinucleated giant cells (arrow) were observed in some tubules (E). Sections of epididymides from several rats exposed to the low-dose combination showed a spermatic granuloma (asterisk) composed of a huge amount of epithelioid-type macrophages and infiltrating lymphocytes surrounding many accumulated spermatozoa (D). In this exposed group of rats, epididymal lumina devoid of spermatozoa often containing multinucleated giant cells (arrows) were observed (F). Bars: 100 µm.

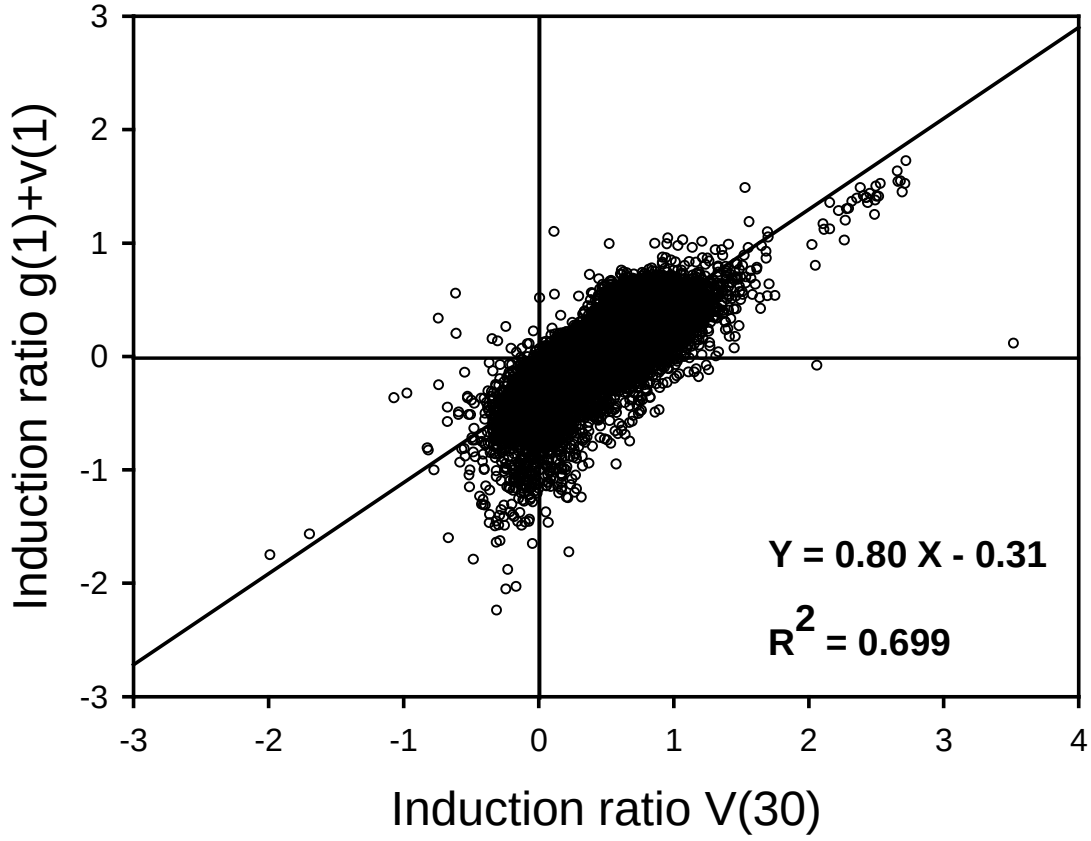

A
B
C
D
E
F

Supplemental Material, Figure 2. Box plot (displaying 10th, 25th, 50th, 75th and 90th percentile values and the mean value as a bold line) of the plasma concentrations of FSH and estradiol in rats aged 80 days.

The concentrations of FSH and Estradiol diverged the furthest from control values in the group g(1)+v(1). *:p<0.05; ⁺:not significant but tendency (p<0.10)



Supplemental Material, Figure 3. Correlation between the logarithms of the induction ratios of V(30)/C and v(1)+g(1)/C in the testis transcriptome. The correlation is 0.836, slightly higher than the value from the correlation matrix that was calculated from the non-transformed values.



References

Delclos KB, Bucci TJ, Lomax LG, Latendresse JR, Warbritton A, Weis CC, et al. 2001. Effects of dietary genistein exposure during development on male and female CD [Sprague-Dawley] rats. Reprod Toxicol 15:647-663.

Fielden MR, Samy SM, Chou KC, Zacharewski TR. 2003. Effect of human dietary exposure levels of genistein during gestation and lactation on long-term reproductive development and sperm quality in mice. Food Chem Toxicol 41:447-454.

Gray LE Jr, Ostby J, Monosson E, Kelce WR. 1999. Environmental antiandrogens: low doses of the fungicide vinclozolin altersexual differentiation of the male rat. Toxicol Ind Health 15:48-64.

Lee BJ, Jung EY, Yun YW, Kang JK, Baek IJ, Yon JM, et al. 2004. Effects of exposure to genistein during pubertal development on the reproductive system of male mice. J Reprod Dev 50:399-409.

Matsuura I, Saitoh T, Ashina M, Wako Y, Iwata H, Toyota N, et al. 2005. Evaluation of a two-generation reproduction toxicity study adding endpoints to detect endocrine disrupting activity using vinclozolin. J Toxicol Sci 30:163-188.

Monosson E, Kelce WR, Lambright C, Ostby J, Gray LE Jr. 1999. Peripubertal exposure to the antiandrogenic fungicide, vinclozolin, delays puberty, inhibits the development of androgen-dependent tissues, and alters androgen receptor function in the male rat. Toxicol Ind Health 15:65-79.

Nagao T, Yoshimura S, Saito Y, Nakagomi M, Usumi K, Ono H. 2001. Reproductive effects in male and female rats of neonatal exposure to genistein. Reprod Toxicol 15:399-411.

O'Connor JC, Frame SR, Ladics GS. 2002. Evaluation of a 15-day screening assay using intact male rats for identifying antiandrogens. Toxicol Sci 69:92-108.

Roberts D, Veeramachaneni DN, Schlaff WD, Awoniyi CA. 2000. Effects of chronic dietary exposure to genistein, a phytoestrogen, during various stages of development on reproductive hormones and spermatogenesis in rats. Endocrine 13:281-286.Shin JH, Moon HJ, Kim TS, Kang IH, Ki HY, Choi KS, et al. 2006. Repeated 28-day oral toxicity study of vinclozolin in rats based on the draft protocol for the "Enhanced OECD Test Guideline No. 407" to detect endocrine effects. Arch Toxicol 80:547-554.

Wisniewski AB, Klein SL, Lakshmanan Y, Gearhart JP. 2003. Exposure to genistein during gestation and lactation demasculinizes the reproductive system in rats. J. Urol 169:1582-1586.